Glycosaminoglycans Production by Cultured Skin Fibroblasts from the Pasini and Cockayne-Touraine Forms of Dominant Dystrophic Epidermolysis Bullosa

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Qualitative and quantitative comparisons of glycosaminoglycans (GAG) production were made on fibroblast lines cultured from the skin of six patients with the Pasini (albopapuloid) form of dominant dystrophic epidermolysis bullosa, six with the non-albopapuloid form (Cockayne-Touraine), eight lines from patients with simplex or recessive dystrophic epidermolysis bullosa and eight lines from normal individuals. A reasonable match of donor age and gender, site, and passage number was achieved. Contrary to an earlier report, the lines from the Pasini group were unexceptional in the amount of GAG they secreted and the proportions of sulfated and nonsulfated GAG showed no consistent difference from the Cockayne-Touraine or control lines. The Pasini lines secreted 77 ± 18 (SEM) μg GAG-uronic acid per 10^7 cells and the Cockayne-Touraine lines 81 ± 12 μg at equivalent cells densities. Sulfated GAG represented averages of 19 ± 4% in Pasini lines, 17 ± 5% in Cockayne-Touraine, and 14 ± 3% in controls. These findings are consistent with current views of albopapuloid lesions as an unreliable clinical sign in epidermolysis bullosa and bring into question the validity of the Pasini entity. *J Invest Dermatol 96:168–171, 1991*

Epidermolysis bullosa is a group of inherited blistering diseases classified into three main subgroups by the level of cleavage within the skin. In dystrophic EB, cleavage is below the basal lamina and the blisters heal, leaving a scar. Two variants of dominantly inherited dystrophic EB are distinguished by the presence (Pasini form) or absence (Cockayne-Touraine form) of albopapuloid lesions, which appear on the lower back of some patients at adolescence [1,2]. In 1979 Bauer and his colleagues [3] reported that fibroblasts grown from the non-lesional skin of seven patients with albopapuloid-dominant dystrophic EB accumulated and secreted excessive amounts of sulfated glycosaminoglycans (GAG) in comparison with fibroblasts from three dominant dystrophic patients lacking the albopapuloid lesions and those from patients with other forms of EB. The excessive production of GAG appeared to be a useful in vitro marker for classifying young patients with dominant dystrophic EB.

There are three reasons why reassessment of GAG production by skin fibroblasts from dominant dystrophic EB is now appropriate. First, we have shown that the amount of GAG released by human skin fibroblasts in vitro is inversely proportional to cell density, which must therefore be controlled for valid comparisons between cell lines [4]. Second, among 26 fibroblast lines from patients with EB and controls, there was increased GAG secretion only in a group of three lines from patients with *non*-albopapuloid–dominant dystrophic EB [5]. Third, there is increasing clinical evidence of variable expression of albopapuloid lesions in families with dominant dystrophic EB and of their appearance in other forms of EB [6,7]. We therefore designed a qualitative and quantitative assessment of GAG production by skin fibroblasts from patients with dominant dystrophic EB.

MATERIALS AND METHODS

Patients and Isolation of Cell Lines Patients with unequivocal family histories of dominant dystrophic EB, whose blisters showed a sublamina densa cleavage on transmission electron microscopy, were classified into two groups according to the presence (dominant dystrophic epidermolysis bullosa, Pasini variant, EBDD-P) or absence (Cockayne-Touraine variant, EBDD-CT) of albopapuloid lesions on the trunk. Biopsies were taken from the forearm or upper thigh under lidocaine anesthesia. The skin was chopped into 1-mm fragments and cultured under coverslips at 37° in Dulbecco-Eagle (DME) medium containing 20% fetal calf serum (FCS), 4 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2/95% air. About 4 weeks later the fibroblast outgrowth was dispersed with trypsin-versene and subcultured in a 25-cm^2 flask. From the second passage the medium contained only 10% FCS and was changed three times weekly. Fibroblast lines from recessive dystrophic and simplex EB and from normal forearm skin (HSF lines) were those isolated previously [8–11] and stored in liquid nitrogen. They were grown in 75-cm^2 plastic flasks in DME me-

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Abbreviations:
DME: Dulbecco-Eagle medium
EB: epidermolysis bullosa
EBDD-CT: dominant dystrophic epidermolysis bullosa, Cockayne-Touraine variant
EBDD-P: dominant dystrophic epidermolysis bullosa, Pasini variant
FCS: fetal calf serum
GAG: glycosaminoglycans
HSF: fibroblasts from normal human skin
S-GAG: sulfated glycosaminoglycans
UA: uronic acid

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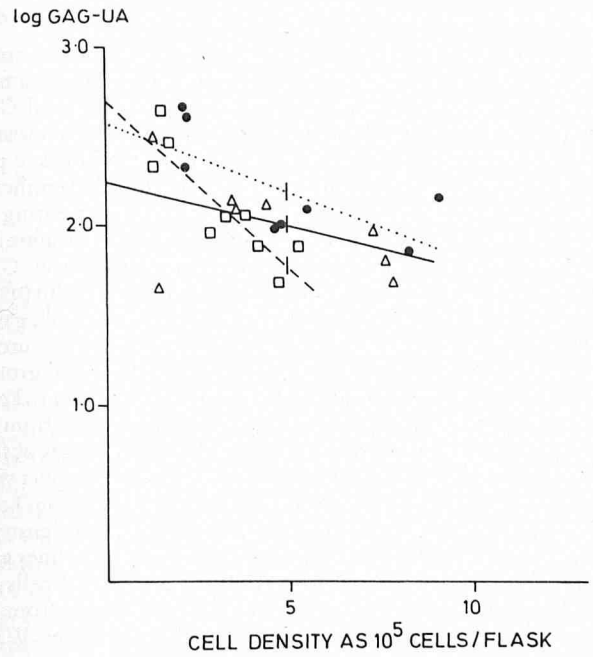

Figure 1. GAG secretion in a single experiment with a line of dominant dystrophic EB fibroblasts *(open triangles, solid line)*, a line of EB simplex fibroblasts *(open square, dashed line)* and HSF fibroblasts from normal skin *(solid circle, dotted line)*. Up to nine cultures were used per line. GAG secretion, as uronic acid (UA), secreted per 10^7 cells per 48 h, is plotted logarithmically against cell density per flask. GAG values at 5×10^5 cells per flask are indicated as intercepts on the regression lines.

dium with 10% FCS plus antibiotics, which was changed three times weekly.

Quantitative Analysis of GAG In the first set of experiments, eight lines of dominant dystrophic EB fibroblasts (four Pasini and four Cockayne-Touraine) were compared with eight other EB lines (four recessive dystrophic and four simplex) and eight lines of normal fibroblasts. For each experiment, one line from each group was selected to give the best three-way match for passage number [3–9], donor age (13–45 years), and gender. From a confluent 75-cm^2 flask, nine subcultures of each line were established, at initial densities of 0.5, 1, and 2×10^5 cells per 25-cm^2 flask (three flasks of each). Medium (7 ml) was changed on days 1, 3, and 4, using the same batch for all cultures on each occasion. On day 6 the cells were trypsinized for cell counts in a Coulter counter and 2 ml of phosphate-buffered saline (PBS) used to rinse the cells was added to the 7 ml of medium. Medium of the same batch was incubated and processed with the samples. They were first dialyzed against three changes of acetate buffer (pH 5), concentrated two-fold with polyacrylamide beads (Lyphogel, Gelman, Northampton, UK), and digested with testicular hyaluronidase (2 mg per sample, 16 h at 37°C). After precipitation of protein in 5% trichloracetic acid, uronic acid in the supernatants was estimated colorimetrically with m-hydroxydiphenyl [12], using glucuronolactone as standard. The uronic acid content of unused medium was subtracted from each value and uronic acid secretion per 10^7 cells was calculated from cell counts and plotted logarithmically as a function of final cell density. Regression lines of log GAG (as uronic acid) on cell density for each cell line were read at 5×10^5 cells per flask and the values assembled into group means. In addition, all the values for cultures in each group were pooled to produce overall regression lines, and the GAG value was again read at a density of 5×10^5 cells per flask. Arithmetic mean GAG-uronic acid values (GAG-UA) for each group were also calculated.

In a second set of experiments, fibroblast lines from four patients with dominant dystrophic EB, two of whom had albopapuloid lesions, were assessed together, because there were no control or other EB lines from donors of similar ages. Cultures were set up at three different densities and the media were collected and processed as before.

Qualitative Analysis of GAG Confluent cultures of six EBDD Pasini fibroblast lines, six EBDD Cockayne-Touraine lines, and six HSF lines in 75-cm^2 flasks were labeled with ^{3}H-glucosamine (100 μCi, 3.7 MBq from Amersham, UK) for 24 h in 10 ml DME medium containing 10% FCS [10]. The same batch of medium was used for all flasks. Medium was then removed and the cell layer was rinsed with 2 ml PBS, which was added to the medium sample. After standing for 10 min in distilled water to swell the cells, the cell layer was scraped off and the cells fragmented by passage through a fine needle. Samples were dialyzed against acetate buffer (pH 5) for 3 d, carrier GAG was added (100 μg each hyaluronic acid, dermatan sulfate, chondroitin-4-sulfate and chondroitin-6-sulfate, from Sigma) and GAG was precipitated at 4°C by adding 1/30th volume of 5% cetyltrimethylammonium bromide. The washed precipitates were digested with pronase at 50°C overnight and reprecipitated with ethanol containing 2% sodium chloride. After a further wash and reprecipitation, the material was dried and redissolved in 100 μl distilled water for electrophoresis.

The GAG was displayed by electrophoresis of 7 μl aliquots on cellulose acetate strips in formic acid/pyridine buffer (pH 3) using approximately 2 mA per cm. The strips were stained in Alcian Blue, dried and cut into 3 mm sections. Each section was added to 1 ml Ultima Gold scintillation medium in a plastic minivial and counted (2×10 min) in a Packard Tricarb scintillation counter. Sample channel ratios showed no differential quenching and results were expressed as ^{3}H cpm per section and plotted graphically. The slow

Table I. GAG Secretion by EB Fibroblasts[a,b]

	EBDD	μg		Other EB μg			HSF	μg
Experiment								
1	EB16	94	CT[c]	EB30	58	S	HSF31	155
2	EB17	105	CT	EB18	80	Dr	HSF26	479
3	EB35	61	P	EB29	174	S	HSF39	162
4	EB37	79	P	EB 5	39	Dr	HSF32	160
5	EB36	31	CT	EB10	26	S	HSF37	70
6	EB25	109	CT	EB 1	155	Dr	HSF23	42
7	EB44	55	P	EB11	99	S	HSF11	118
8	EB41	162	P	EB 2	22	Dr	HSF22	32
Mean ± SEM		87 ± 14			82 ± 20			152 ± 50
Subgroup[c] means (n)	EBDD.CT (4)	85		EBDr (4)	74		HSF (8)	152
	EBDD.P (4)	89		EBS (4)	89			

[a] Read from linear regression of log GAG-uronic acid on cell density at 5×10^5 cells per flask (7 to 9 cultures per line).
[b] Differences between the three main groups were not statistically significant (Student t and Wilcoxon rank tests).
[c] Abbreviation for EB subgroups: CT, Cockayne-Touraine; P, Pasini; S, Simplex; Dr, recessive dystrophic; HSF, controls.

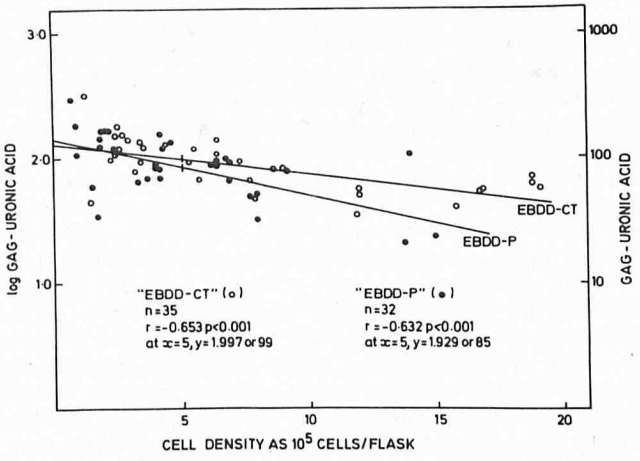

Figure 2. GAG secretion by lines of skin fibroblasts from dominant dystrophic EB plotted as a function of cell density. Four lines were from patients with albopapuloid lesions (EBDD-P, *solid circle*) and four were from patients without lesions (EBDD-CT, *open circle*), with up to nine cultures per line. Intercepts on the regression lines at 5×10^5 cells per flask are indicated.

high peaks present in all samples were regarded as hyaluronic acid and the smaller amount of faster-migrating material as sulfated GAG (S-GAG). The assumptions were tested by incubating portions of a GAG sample (from the medium of a Cockayne-Touraine fibroblast line) with streptococcal hyaluronidase and chondroitinase ABC (Sigma) before electrophoresis. To calculate final percentages of sulfated GAG, cpm values from cell and medium preparations were added together to give the definitive value for each cell line.

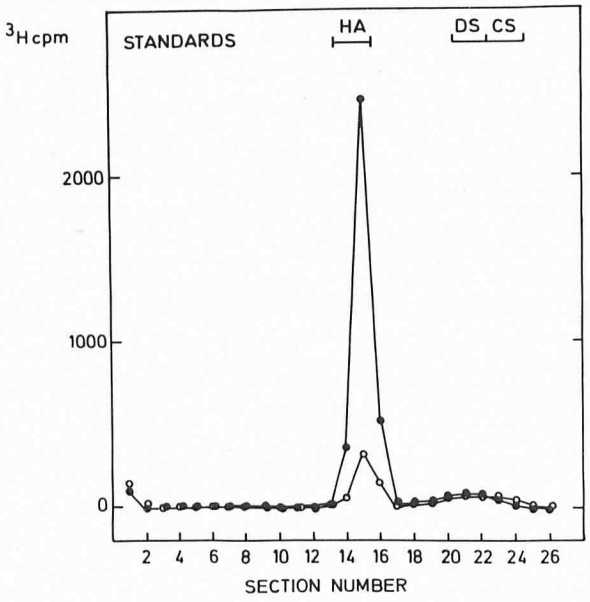

Figure 3. Electrophoretic display of ^{3}H-GAG from fibroblasts cultured from a patient with EBDD. Similar preparations were made for six patients with EBDD-P, six with EBDD-CT and for six normal controls (HSF) to produce the data in Table II. Migration on cellulose acetate was from left to right. The sharp peaks in sections 14 to 16 were regarded as hyaluronic acid (85% of total) and the radioactivity in sections 17 onwards as sulfated GAG (15% of total). The positions of non-radioactive standards are indicated: HA (hyaluronic acid); DS (dermatan sulfate); CS (chondroitin sulfate). Separate traces are shown from cells *(open circles)* and medium *(solid circles)*.

RESULTS

Quantitative Findings Figure 1 shows the results of a single experiment in the first series and Table I summarizes the data from the eight experiments. There were wide variations in total GAG output (cf. HSF23 and HSF26) within each group but no consistent difference between the groups. The tendency for HSF lines to produce more GAG than the EB lines was not statistically significant. Within the EB groups, the subgroups of four lines representing the Cockayne-Touraine and Pasini forms of dominant dystrophic EB, recessive dystrophic, and simplex EB were similar in mean GAG output. Regressions prepared from all the data in each group (maximum 72) pooled together, and read at 5×10^5 cells per flask, gave a similar overall result, namely, 93, 100, and 128 μg GAG-uronic acid from the dominant dystrophic, other EB and control groups, respectively. Regression lines for the Pasini and Cockayne-Touraine subgroups were similar (Fig 2); if anything, fibroblasts from the Pasini form gave slightly lower values. In the second series of four fibroblast lines from dominant dystrophic EB, GAG outputs were low despite generally low cell densities. The two Cockayne-Touraine lines gave 167 ± 18 (SEM) μg GAG-UA at the mean density of 2.03×10^5 and 87 ± 8 μg at 3.83×10^5. The two Pasini lines gave 81 ± 14 μg at 3.56×10^5 and 69 ± 10 μg at 5.69×10^5 cells per flask. These data contain no suggestion of greater output from the Pasini fibroblasts, and support the findings in the main series. When the second set of data is corrected for cell density (i.e., read from linear regressions at 5×10^5 cells per flask) and pooled with the first set, the mean value for the six Cockayne-Touraine lines becomes 81 ± 12 μg GAG-UA and for the Pasini lines 77 ± 18 μg GAG-UA, and the difference is not statistically significant ($t = 0.18$).

The general distribution of ^{3}H-GAG conformed to previous descriptions [3,10,13,14]. About 90% was present in the culture medium where the display was dominated by a sharp peak comigrating with the hyaluronic acid standard. Ahead of this was more dispersed material corresponding to the chondroitin sulfate and dermatan sulfate standards. Streptococcal hyaluronidase removed the slow peak, confirming its identity as hyaluronic acid. Chondroitinase ABC removed all the faster-sedimenting radioactivity, showing it to be chondroitin sulfate or dermatan sulfate and leaving no residue to suggest the presence of heparan sulfate. Percentages of S-GAG were higher in the cells than in the medium, and varied considerably (5–38%) between cell lines when expressed as total S-GAG (cells plus medium) (Table II). There were, however, no consistent differences between the three groups of cell lines and their overall mean values for S-GAG did not differ significantly.

DISCUSSION

This assessment of GAG production in fibroblasts from dominant dystrophic EB failed to show any exceptional features of cell lines from 6 patients with the Pasini (albopapuloid) form in comparison with six fibroblast lines from patients with the Cockayne-Touraine (non-albopapuloid) form. Fibroblasts from dominant dystrophic patients were also indistinguishable from those derived from patients with simplex or recessive dystrophic EB. This therefore fails to confirm the earlier findings [3,5].

Bauer's comparison [3] of seven lines from Pasini patients with three from Cockayne-Touraine patients compared synthesis and secretion of sulfated GAG only. Because hyaluronic acid, which is not sulfated, forms the bulk of fibroblast GAG, it was possible that the quantitative data in Table I for total GAG masked a shift from hyaluronic acid to sulfated GAG production, which might be compatible with the earlier findings. However, this possibility was dispelled by the qualitative analysis (Table II): the percentages of sulfated GAG varied considerably but with no trend distinguishing either subgroup.

Possible explanations for the discrepancy between earlier and current findings include the allowance for differences in cell density, which had a central role in our assessment but was not made in Bauer's study. Another problem is that the number of fibroblast lines that can be examined in any one study is small and as the characteristics are often highly variable, unrepresentative findings

Table II. Percentage of Sulfated ^{3}H-GAG in Fibroblast Cultures[a]

EBDD − P	EBDD − CT	HSF
39	38	24
22	20	19
15	19	17
15	10	16
13	8	5
9	6	4
Mean ± SEM 18.8 ± 4.4%	16.8 ± 4.9%	14.2 ± 3.3%

[a] Each value is from a different fibroblast line and represents total GAG production (cells plus medium). Differences between means were not significant (Student t test: for EBDD − P versus HSF, $t = 0.85$, $p > 0.2$).

are always a possibility. The reservation applies to the present study too, but other, so far less systematic, studies of GAG production by EB fibroblasts, have also failed to find a GAG abnormality in the Pasini group [15,16]. It is also important to question the suggestion [3] that the level of GAG production is a genetic trait. Oakley's survey of fibroblast lines [5] recorded high secretion rates in three dominant dystrophic (CT) lines but these, EB16, EB17, and EB25 in Table I, were unexceptional when reassessed after storage for 5 years in liquid nitrogen.

Apart from the use of single batches of medium for the cultures being compared at any one time, no special precautions were taken to control pH in these experiments. Change in intracellular catabolism and accumulation of GAG has been reported to be pH-dependent [17], but in our earlier work [4] the pH of the medium was stable over a 6-d period using an identical regime to that of the quantitative assays in the current study. Secreted GAG represents more than 90% of the total in fibroblast cultures [4] and was not affected by pH [17]. Any variation in medium pH that occurred in this work would have affected all the cultures and does not seem a likely explanation for the differences in GAG output evident between cell lines in Table I.

Earlier reports that the Pasini and Cockayne-Touraine forms of dystrophic EB could be distinguished clinically by the severity, distribution, and age at onset of the blistering, and by the ultrastructural distribution of anchoring fibrils [18,19], are now in doubt. Morphometric studies have not confirmed a distinction based on anchoring fibril morphology or distribution [20]. The justification for the Pasini entity therefore rests on the presence of the albopapuloid lesions, although no connection has been established between the appearance of lesions on the trunk and the blistering elsewhere on the body. Albopapuloid lesions appear in a proportion of patients with dominant dystrophic EB; they occasionally vary within families and have now been reported in other forms of EB by several observers around the world [7,20,21]. It seems likely that patients with dominant dystrophic EB will be regarded as a single group in the future, with advantages for investigation and treatment. Advances in genetic analysis of EB should provide confirmation for this approach in the near future [22].

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